SHIMADZU

MP 471

Rapid Differentiation Between Mixed A1/A2 and A2 Only Cow Milk Using MALDI-TOF-MS

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1. Overview

Samples of mixed A1/A2 milk and A2 only cow milk were analyzed using MALDI-TOF-MS utilizing trypsin digestion to distinguish a single amino acid difference between the products. On-plate digestion of resulting milk proteins was optimized to allow for detection of both extracted milk proteins and diluted raw milk samples. A2 only milk spiked with varying concentrations of mixed A1/A2 milk were analyzed to determine the limit of detection (LOD) using MALDI-TOF-MS.

2. Introduction

The A2 only milk market is forecasted for high growth, some estimates peg annual growth rates of 21.7% until 2029[1].The difference between mixed A1/ A2 and A2 only cow milk is the lack of A1 β-casein in the later. Several studies suggest benefits for A2 only milk over mixed A1/A2 milk including better digestibility. With these benefits, A2 only milk commands a premium. Therefore, tests to differentiate mixed A1/A2 from A2 only milk are important for quality control or determining adulteration. Related tests on the market are primarily genetics based and focus on determining a cow's ability to produce A2 only milk. In this work mixed A1/A2 and A2 only milk samples are differentiated by MALDI-TOF-MS based on β-casein fragments.

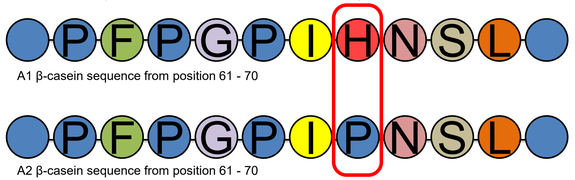

Figure 1: Amino acid sequence of A1 and A2 β-casein from position 61 - 70

3. Methods

Both mixed A1/A2 milk and A2 only milk were locally sourced from grocery stores.

Milk protein extraction[2]: 15 mL of raw milk was centrifuged at 4900 rpm for 10 min, and the fat plug was removed. The pH of the supernatant was adjusted to 4.6 and then centrifuged. Pellet was washed with acidic water, centrifuged then washed in basic water and centrifuged a second time. The resulting washed pellet was used for further analysis.

In solution trypsin digestion: Trypsin/Lys-C at a 25:1 protein to protease ratio was added to a 50 mg/mL solution of milk protein extract in 50 mM Tris HCl (pH 8.0) and incubated overnight at 37ºC. Addition of trifluoroacetic acid (TFA) to a final concentration of 0.7% terminated the digestion. Afterwards samples were centrifuged at 10,000 rpm for 15 min.

On plate trypsin digest: Adapted from Harris & Reilly 2002[3]. Trypsin was spotted and dried followed by 5 mg/mL of milk protein extract in 50 mM Tris HCl (pH 8.0) . The plate was incubated at 37°C in a humid environment for 15 minutes. Digestion was terminated with the addition of 1% TFA and dried.

MS conditions (MALDI 8030)

Laser Rep. Rate (Hz): 50
Polarity: Positive Mode
Laser Power: 50-60
Ion Gate (Blanking): 650
Pulsed Extraction: 5400

Figure 2: MALDI 8030 MALDI-TOF MS.

4. Results

A2 only milk and A1/A2 mixed milk are differentiated by the presence of A1 β-casein. These were distinguished by analyzing milk samples for the presence of A1 β-casein proteolytic fragment (49-97) as it is the only tryptic fragment that has a different mass from its A2 β-casein proteolytic fragment counterpart as it contains the amino acid on the 67th position. Figure 3 contains the ProteoMass detailing the theoretical tryptic fragments of A1 and A2 β-casein.

A) A1 β-casein

PeptideMass

The entered sequence is:

```
        10        20        30        40        50        60
RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY

        70        80        90       100       110       120
PFPGPIHNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE APAPKHKEMP FPKYPVEPFT

       130       140       150       160       170       180
ESQSLTLTDV ENLHLPLPLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY

       190       200
PQRDMPIQAF LLYQEPVLGP VRGPFPIIV
```

Mass	Position	Peptide Sequence
6363.3300	114-169	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK
5360.2818	49-97	IHPFAQTQSLVYPFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK
2647.8494	2-25	ELEELNVPGEIVESLSSSEESITR
2187.6046	187-202	DMPIQAFLLYQEPVLGPVR
1983.0068	33-48	FQSEEQQQTEDELQDK
830.8617	171-183	AVPYPQR
780.9055	170-176	VLPVPQK
748.9149	108-113	EMPFPK
742.9360	203-209	GPFPIIV
646.7792	100-105	EAMPK

B) A2 β-casein

PeptideMass

The entered sequence is:

```
        10        20        30        40        50        60
RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY

        70        80        90       100       110       120
PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE APAPKHKEMP FPKYPVEPFT

       130       140       150       160       170       180
ESQSLTLTDV ENLHLPLPLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY

       190       200
PQRDMPIQAF LLYQEPVLGP VRGPFPIIV
```

Mass	Position	Peptide Sequence
6363.3300	114-169	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK
5320.2572	49-97	IHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK
2647.8494	2-25	ELEELNVPGEIVESLSSSEESITR
2187.6046	187-202	DMPIQAFLLYQEPVLGPVR
1983.0068	33-48	FQSEEQQQTEDELQDK
830.8617	171-183	AVPYPQR
780.9055	170-176	VLPVPQK
748.9149	108-113	EMPFPK
742.9360	203-209	GPFPIIV
646.7792	100-105	EAMPK

Figure 3: Tryptic fragment results generated from PeptideMass for **A)** A1 β-casein and **B)** A2 β-casein. Proteolytic fragment (49-97) masses and 67th position amino acid for both proteins were highlighted

Milk protein extract for both A2 only and A1/A2 mixed milk underwent an in-solution tryptic digest. Digests were spotted onto a stainless steel MALDI target with SA as the matrix show differentiation between A2 only and A1/A2 mixed milk (Figure 4)

To reduce sample preparation time an on-plate trypsin digestion was adapted from Harris & Reilly 2002. Similar results as the in-solution trypsin digest was observed, while reducing the digestion time from overnight to 30 min (Figure 5).

To further reduce sample preparation time the protein extraction step was substituted with diluting raw milk in 50 mM Tris HCl. Afterwards the on-plate digest procedure was performed. Similar results as the in-solution trypsin digest was also observed (Figure 6).

Determining the adulteration of A2 only milk with A1/A2 mixed milk was studied using A2 milk spiked with various concentrations of A1/A2 mixed milk. Two different matrices: sinapinic acid (SA) and SA/ Alkylated Trihydroxyacetophenone (ATHAP)[4] were tested.

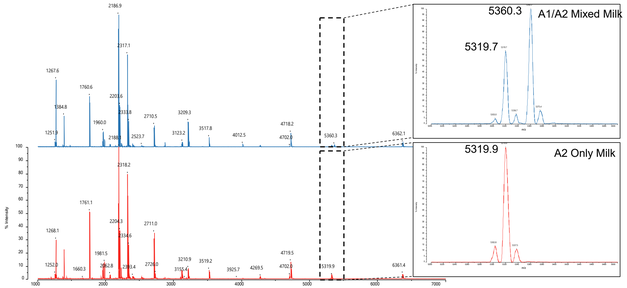

Figure 4: Mass spectrum of A2 only milk (red) and A1/A2 mixed milk (blue) of milk protein digest after an in-solution tryptic digest using SA as the matrix. Inserts magnified the 5200 – 5500 m/z range for both samples.

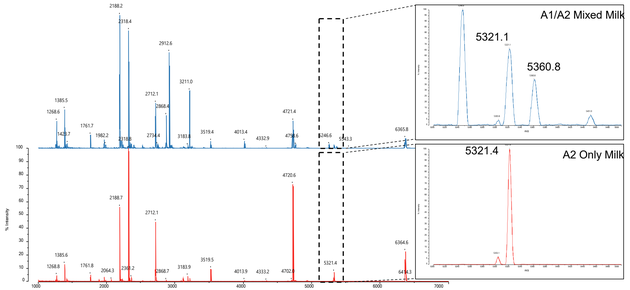

Figure 5: Mass spectrum of A2 only milk (red) and A1/A2 mixed milk (blue) of milk protein digest after an on-plate tryptic digest using SA as the matrix. Inserts magnified the 5200 – 5500 m/z range for both samples.

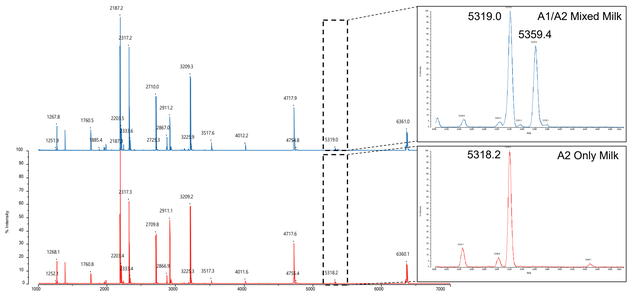

Figure 6: Mass spectrum of A2 only milk (red) and A1/A2 mixed milk (blue) of diluted milk after an on-plate tryptic digest using SA as the matrix. Inserts magnified the 5200 – 5500 m/z range for both samples.

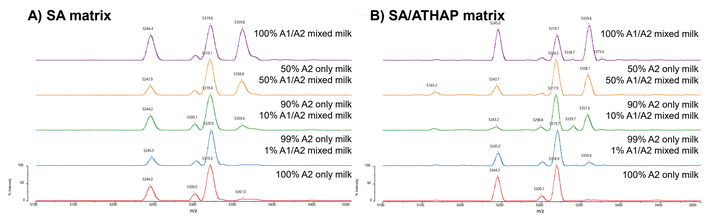

Figure 7: Mass spectrum of milk protein extract after in solution tryptic digestion of A2 only milk blended with various volumetric concentrations of A1/A2 mixed milk. **A)** uses SA as the matrix while **B)** uses SA/ATHAP as the matrix. Red, blue, green, yellow, and purple spectra corresponds to concentrations 100%, 99%, 90%, 50%, and 0% A2 only milk samples respectively.

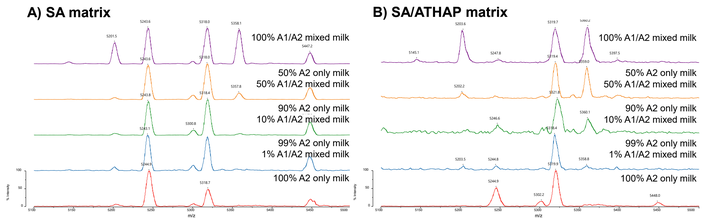

Figure 8: Mass spectrum of diluted milk after an on-plate tryptic digestion of A2 only milk blended with various volumetric concentrations of A1/A2 mixed milk. **A)** uses SA as the matrix while **B)** uses SA/ATHAP as the matrix. Red, blue, green, yellow, and purple spectra corresponds to concentrations 100%, 99%, 90%, 50%, and 0% A2 only milk samples respectively.

5. Conclusions

MALDI-TOF-MS can be used to easily distinguish between mixed A1/A2 and A2 only cow's milk from a tryptic digestion of the A1 and A2 β-casein proteins present in milk. The changing of matrix solution from only SA to a mixture of SA and ATHAP increased sensitivity of A1 β-casein for detecting presence of mixed A1/A2 milk spiked in A2 only milk.

6. References

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The authors declare no competing financial interest.

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